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High Resolution Melt Analysis of Samples with Differential DNA Methylation to Identify Tissue Source of Origin

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HIGH RESOLUTION MELT ANALYSIS OF SAMPLES WITH DIFFERENTIAL DNA METHYLATION TO IDENTIFY TISSUE SOURCE OF ORIGIN

by

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ABSTRACT

HIGH RESOLUTION MELT ANALYSIS OF SAMPLES WITH DIFFERENTIAL DNA METHYLATION TO IDENTIFY TISSUE SOURCE OF ORIGIN

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In Forensic Science casework, identifying the source of a tissue can assist in crime scene reconstruction. Currently, presumptive testing methods to identify tissue type are utilized in crime scene laboratories, but there is a need for a more reliable confirmatory test for tissue type identification. High Resolution Melt (HRM) analysis is an innovative technology that has the potential to determine tissue types through variations in DNA methylation patterns. Recently, DNA methylation patterns have been found to correspond with specific tissue types in particular regions of DNA. Two markers, B_SPTB_03 and DDX4 have been effective in differentiating sperm from other tissue types, and two markers, cg-6379435 and cg-8792630 have been effective in differentiating blood from other tissue types. This study was conducted to examine if HRM analysis could differentiate tissue type based on DNA methylation patterns. For the two sperm markers examined, B_SPTB_03 had a melting temperature of 3.5℃ higher than the other tissue types tested (skin epithelial, vaginal epithelial, blood, and buccal), and DDX4 had a melting temperature that was 3.0℃ lower than the other tissue types. In addition, both of the blood markers, cg-06379435 and cg-08792630, were found to have melting temperatures approximately 1.0℃ higher than the other tissue types. The differentiation in melting temperature depends on three factors: the number of CpG sites

available for study, the amount of methylation differences between two tissues, and the percentage difference in the DNA sequences between two tissue types.

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CHAPTER I

INTRODUCTION

Biological evidence found at crime scenes is becoming an increasingly useful tool in the fields of Criminal Justice and Forensic Science. The type of sample found assists in recreating the crime scene and also in identification of a suspect or victim. Present technologies that test for the type of biological material present are mainly presumptive testing methods (Abacus Diagnostics, Inc., 2014). Furthermore, the equipment and materials needed to perform these tasks are time consuming and expensive.

Although DNA technology is a recent development, it has expanded with innovative applications in Forensic Science. This expansion began with the development of Restriction Fragment Length Polymorphism (RFLP) in the 1980s. Dr. Alec Jeffreys was the first to use this technology to determine Variable Number of Tandem Repeats (VNTRs) to differentiate between individuals. This was only the start of the momentum of DNA technology as it was followed by Dr. Kary Mullis's invention of Polymerase Chain Reaction (PCR) in 1985. This revolutionary technology allows scientists to quickly amplify small amounts of DNA to a finished product of millions of copies of DNA. Furthermore, it led to the usage of Short Tandem Repeats (STRs) that are currently still a prominent fixture in DNA typing. STRs are an improved method of identification from VNTRs due to the variety of alleles present in the population and the ease of amplification using PCR. Currently, 13 STR loci are used for human identification along with Amelogenin, which is a gender identification locus (Brief History of Forensic DNA Typing, 2010).

Biological samples are also useful in crime scene reconstruction. They can help piece together what events occurred by the presence or absence of certain tissue types. Recent practices to detect tissue types vary based on the source you are trying to detect. To determine if blood is present at the scene, several testing methods are available. *BLUESTAR*® offers several reagents that can show bloodstains, even after the stain has been washed out. This test can be used for identification of blood, but if the sample found at a crime scene is negative for blood, there is no other test offered that could identify what type of sample it is. The Kastle-Meyer Phenolphthalein Test is also utilized to detect the presence of blood at crime scenes. Although helpful in determining if hemoglobin is present, the Kastle-Meyer test is not human-specific so it can yield a false positive for use in human blood identification. Luminol based tests are also used for presumptive testing for blood. However, this test can only indicate that blood might be present and other substances such as bleach can also give false-positive results (James, Nordy, & Bell, 2002).

A species origin testing method for blood is Ouchterlony immunodiffusion test. This test can determine if a blood sample is from a human or animal through antibody reactions. The ouchterlony test must be performed and interpreted by an experienced scientist due to the complexity and positivity of the test. Also, it may take up to 72 hours for this test reaction to take place (Serology-Blood and other bodily fluids, 2014).

The presence of semen at a crime scene provides beneficial information. One of the testing methods available for semen is the Acid Phosphatase Test, commonly referred to as the AP spot test. This presumptive test relies on a color indicator of the chemical reaction of the enzyme acid phosphatase with Brentamine Fast Blue. The test is

classified as a presumptive test because other bodily fluids such as vaginal secretions have amounts of this enzyme that can provide a false positive for semen (Noureddine, 2011).

Currently, testing for the presence of semen also includes the $ABACard^{®}$ p30 test, microscopic detection, and the usage of alternate light sources (ALS). The $ABACard^{®}$ p30 tests for the p30 antigen, also known as the prostate specific antigen that is present in seminal fluid. The $ABACard^{®}$ p30 is a promising test, and requires proteins that are not degraded for maximum effectiveness (Koch et al., 2015). Often times, samples found at crime scenes are degraded and therefore, the $ABACard^{®}$ p30 test can be problematic.

All of these tests to identify the tissue source give scientists valuable information about the sample, but also have a downside. All the tests cannot be run simultaneously and they do not use the same reagents and supplies. Also, a majority of the tests are presumptive and may not give a definite answer to the presence of the specific tissue types in question. They require a larger amount of sample, which is impractical for crime scenes.

A currently used method of identification utilizes DNA methylation patterns to identify tissue type. DNA methylation is a type of epigenetic modification. Epigenetics includes the changes in gene activity and expression in a cell that do not alter the DNA sequence. Instead, the changes are made by DNA methylation or histone modifications. Methylation can appear on the 5' carbon of the cytosine (C) that has a guanine (G) base that follows. Between the 'C' and 'G' consecutive bases, a phosphodiester bond links the two, creating the name 'CpG'. Studies have shown that sperm have been differentiated

from other bio fluids based on DNA methylation patterns. Six markers, including B_SPTB_03 and DDX4 have been proved to have differentially methylated in semen from other tissue types. B_SPTB_03 was found to be hypermethylated in semen samples while all other tissue types were hypomethylated. In addition, a difference was found for DDX4, which was hypomethylated in semen, whereas the other tissue types were hypermethylated (Balamurugan, Bombardi, Duncan, & McCord, 2014). Studies such as this are promising for identification of bio fluid source and can still be improved on.

Another study that examines methylation patterns includes research that has distinguished blood samples from saliva, semen, and vaginal secretions based on DNA methylation levels at specific markers (Park et al., 2014). This study selected markers that were hypermethylated in one body fluid, had a large methylation difference, and was practical for pyrosequencing. Two markers, cg-06379435 and cg-08792630 were found to have statistically different methylation patterns for blood in comparison to the other sampled tissue types.

High Resolution Melt (HRM) analysis is a powerful method that can detect single nucleotide differences in strands of DNA by analyzing the level of separation of double stranded DNA through the temperature that it becomes denatured at. The process of HRM begins with the amplification of the target region of DNA. Polymerase Chain Reaction (PCR) is a method that exponentially increases the targeted DNA region by cycling the DNA through various temperatures to separate, anneal, and extend the sequence. This process can be repeated as many as 25 cycles to create a large amount of DNA. During the PCR process, unique dyes known as intercalating dyes are added to the DNA sequence. Intercalating dyes only fluoresce while incorporated in double stranded

DNA. If the DNA is separated into single strands, the dye will cease to fluoresce (High Resolution Melt Analysis, 2014).

There are multiple intercalating dyes used in HRM technologies today. Some examples of these dyes are LC Green, SYTO® Dye, EvaGreen®, and Chromofy™ which are commonly used in HRM analysis for methylation analysis. Variance among the dyes is primarily how they bind to the nucleotides and how their fluorescence affects the result analysis (High Resolution Melt Dyes, 2014).

Following the PCR process, the amplicon is slowly heated from 60℃ to 95℃. During this gradual heating, the double stranded DNA begins to separate and loses its fluorescence. As an increasing amount of DNA is separated, there is a decrease in fluorescence. High Resolution Melt Analysis is used when a melt curve is created which plots the level of fluorescence versus the temperature. A HRM curve can detect single nucleotide differences between alleles via the two diverse curves that will be produced (High Resolution Melt Analysis, 2014).

Detecting differences in two HRM curves can be applied in multiple applications: detecting single nucleotide mutations and sequencing differences, DNA mapping, species identification, zygosity testing, DNA fingerprinting, methylation analysis via GC content, and discriminating sequence length and strand complementarity. Detection is possible through the highly powerful ability to record differences in the target DNA's disassociation (High Resolution Melt Analysis, 2014).

Understandably, HRM Analysis has endless applications and just as many advantages. The technology that runs and analyzes samples undergoing HRM is a cost-effective one. Genotyping technologies such as sequencing and Taqman SNP typing are

high-priced due to materials, equipment, and other factors. However, HRM is an economical method compared to other genotyping methods. HRM also is a straightforward technology that can perform both the PCR and HRM Analysis in one instrument. This reduces extra expenses, time, and creates a simpler workflow. In addition, HRM Analysis uses fewer reagents than typical genotyping technologies. It is only necessary to create the PCR reaction volume for each sample to be analyzed and eliminates the need for solvents and electrophoresis gels. Lastly, HRM is a fast and powerful technology. It can perform highly accurate genotyping to a hefty quantity of samples in a short amount of time (High Resolution Melting Analysis, 2014). Clearly, HRM is a technology that can be incorporated more often in Forensic Science laboratories.

Aims and Objectives

The aim of this research was to determine if High Resolution Melt Analysis can detect tissue source of DNA based on tissue specific differential DNA methylation patterns. Two markers, B_SPTB_03 and DDX4 were tested using HRM melt curve analysis to see if this new and innovative technology could be used to differentiate sperm from the other tissues. Two other markers, cg-06379435 and cg-08792630 were also used to differentiate blood from other tissues.

CHAPTER II

REVIEW OF LITERATURE

Presumptive DNA Testing Methods

Current tests such as the Kastle-Meyer Test used for the identification of the presence of blood have downfalls. However, they are still routinely used in the crime laboratory. One problem of these tests is that they can both give false positive if reacting with common compounds (Mullen, 2009). In addition, both these tests and other presumptive tests are dependent upon the interpretation of the qualitative information from the analyst. Also, it is important to note that some bodily fluids do not have available testing methods such as vaginal fluid (Prinz, Tang, Siegel, Yang, & Zhou, 2011). The assays currently used to test for semen, such as AP test and PSA testing, have limitations, including low specificity or sensitivity and little sample left after the test is completed (James et al., 2002). Another downfall for protein-based assays is that biological evidence at a crime scene is usually degraded by the environment and will not yield an accurate result (Noureddine, 2011).

Currently, work involving messenger RNA (mRNA) has been showing some promise in identifying specific gene expression in human organs. However, using mRNA to detect tissue type is not without downfall. mRNA expression levels are not detectable in all organs and certain mRNA expression levels such as NR2E3 mRNA are similar in both female and male reproductive systems as well as the salivary gland, making the determination between sperm and vaginal epithelial tissue types difficult (Nishimura, 2004). Other markers were found that have the potential to differentiate between tissue types as well (Nishimura & Naito, 2005). Additionally, RNA is not as

stable as DNA. This instability can prove to be difficult with samples that are not preserved. Samples are often degraded when found at crime scenes, limiting RNA's usefulness in forensic applications.

In recent years, Micro RNA (miRNA) has also been studied to identify tissue-specific expression levels. miRNA is involved in RNA silencing and gene expression in post-transcriptional regulation. This has led researchers to believe that miRNA can be used to identify tissue source from biological matter. The current research on this possibility has expanded, but due to the fact that several miRNA work together for each regulatory network, the research has hit a plateau for its possibility in tissue identification research (Guo, Parker, Yang, & Hu, 2014). RNA technology, although beneficial, is a less common practice in forensic biology laboratories. Therefore, developing a DNA-based tissue identification method for biological material would be a more promising solution. In addition, scientists are accustomed to using DNA, so a DNA based technology would prove most useful in forensic science laboratories.

The existing practice for human identification involves the amplification of 13 CODIS STR loci as well as Amelogenin marker. A commonly used kit to perform this PCR is the $AmpFlSTR^{®}Identifiler^{®}$ PCR Amplification kit from Applied Biosystems (Foster City, CA) (Collins et al., 2004).

DNA Methylation Testing Methods

CpG islands are defined as "regions that are >200bp with >50% GC content and have a ratio of >0.6 of observed GC dinucleotides compared to the expected number based on GC content" (Kent et al., 2002). Eckhardt et al (2006) studied the DNA methylation profiles on the CpG islands focusing primarily on chromosomes 6, 20, and

22. In this study, they compared samples from 2 age groups to determine if there were differences in the group with a mean age of 26 from the group with a mean age of 68. To determine if there was a sex-related marker, they choose samples from the same age group but belonging to different sexes. This study did not find any significant results under the experimental conditions, leading to the conclusion that methylation patterns will only have correlation to specific locations on the genome and specific tissues (Eckhardt, Lewin, Cortese, Rakyan, & Attwood, 2006).

In a recent study by Balamurugan et al. (2014), spermatozoa were differentiated from other tissue types commonly found at crime scenes. In this study, 6 markers were proven to identify tissue-specific DNA methylation patterns for semen, including DDX4 and B_SPTB_03. The goal of this study was to identify semen-specific markers based on comparison of DNA methylation profiles differing on tissue type for advancing the field of forensic science. All six markers had significantly different DNA methylation patterns for semen compared to the other tissue types. DDX4, for example, plays a role in the process of spermatogenesis. Therefore, research suggests that this marker should be highly expressed in semen but not in other tissue types. The DDX4 site showed a range of 3-6% methylation for sperm samples and 56-100% methylation range for the other tissue types (Balamurugan et al., 2014). The study confirms this by showing that sperm samples were hypomethylated at this marker whereas the other tissue type samples were not. Another marker, B_SPTB_03 was originally thought of as a potential blood marker based on previous research (Haas, Klesser, Maake, Bar, & Kratzer, 2009) and that it is part of the β-spectrin family, a family of genes that assists with the stability of erythrocyte membranes (Haas et al., 2009). However, results from Balamurugan et al.

(2014) study indicated differently. This study showed that this marker had methylation values in the range of 77-100% in sperm, and the other tissue types ranged from 1-13% methylation (Balamurugan et al., 2014).

A second study that used DNA methylation as a marker for forensic science applications was performed by Park et al. (2014). In their study, they studied genome-wide DNA methylation profiling using Illumina Human Methylation 450K bead array. This technology identified over 450,000 CpG sites. They then narrowed those sites down to identify almost 3,000 markers specific to a certain body fluid. Following this task, they used pyrosequencing to test 8 markers, 2 tissue-specific markers for each body fluid tested. Their results of the pyrosequencing study showed several markers for blood, including cg-06379435 and cg-08792630 that were "strongly hypermethylated in the target body fluid compared with the other body fluids" (Park et al., 2014). Also, this study found some age related markers in the process of narrowing down markers for tissue-specific study. DNA methylation will be altered during the course of aging, so it is important to only find markers that will be reliable markers for singling out a body fluid regardless of age.

It is known that global DNA methylation in human tissue decreases over time. However, some individual genes become more methylated, to the point of hypermethylation, with age. Therefore, methylation changes must be a result of other regulatory components, and not a sign of deterioration in DNA. Recently, an age-related study by Yi, Jia, Mei, Yang, and Huang (2015) found 3 markers, USP11, NOP14, and ZIC5 that displayed significant differences in methylation levels between samples between their labeled 'junior' and 'senior' groups. In addition, this study found that all

three markers could accurately identify an unknown individual's age with accuracy of up to 4 years difference from the predicted and correct age (Yi et al., 2015).

HRM DNA Testing Methods

Dr. Bates recently published an article in *Genetic Engineering and Biotechnology News* that described how Melting Point Analysis has the resolution to distinguish Single Nucleotide Polymorphisms (SNPs) in DNA (Bates, 2014). It reviewed the process of HRM and that the melting temperature (T_m) of PCR products can vary based on the length and DNA sequence. The bases "G" and "C" have 3 hydrogen bonds connecting them whereas the bases "A" and "T" have only 2. This small difference will affect the melting temperature in which the bond will break by requiring a higher melting temperature for DNA with larger amounts of GC content. The melting temperature difference can then pinpoint a difference in a SNP based on a change in the T_m.

The T_m is defined as the point where 50% of the DNA is in the double stranded state and 50% is in the single stranded state. By analyzing differences in the T_m, and by analyzing the melting curve graph, differences in the genetic makeup can be identified. In her study, Bates used two synthesized 100-base single stranded DNA with a purposeful mutation, a mutant "A" in place of the "G", placed in one of the strands. Both single stranded templates were replicated so a total of 6 separate HRM analyses with 4 different kits could be run. The results showed a large increased shift in T_m between the two strands due to the "G" nucleotide base change to the "A" nucleotide base. Each run showed the same result: "In each case, the six replicates for each template type were clearly distinct" (Bates, 2014, p. 25).

Alvarez-Sandoval, Manzanilla, and Montiel (2014) studied another application for HRM Analysis: Sex Determination. In their study, they explored the possibility that highly degraded samples could have a huge benefit from HRM Analysis with little drawback. Because their study involved samples that were susceptible to contamination and contained small amounts of degraded sample, using HRM was a suited choice. The melt curves were differentiated from the 3 base pair deletion that occurs on the X Chromosome resulting in a difference from Y Chromosome. The laboratory followed in-house protocol for the extraction and used a Roche Applied Science Kit for the reaction, using $2\mu L$ of template DNA, with the master mix, primers, and EvaGreen® Dye to ensure there was no PCR interference and that their PCR signal and melt peak would be clear (Alvarez-Sandoval et al., 2014). The EvaGreen® Dye was used because it is an saturating dye which do not interfere with PCR reactions, even if they used at the largest level of saturation which gives the maximum fluorescence (High Resolution Melt Dyes, 2014). Their results showed distinct differences in the curves for the male and female samples permitting them to identify the sex of the samples. The HRM results were confirmed by analyzing the DNA sequences to detect the 3 base pair difference. (Alvarez-Sandoval et al., 2014).

The possibilities of HRM application for detection of single nucleotide polymorphism (SNP) were examined by Brunstein (2014). The curve shapes produced by the HRM differentiate between the SNP and the original sequence, providing two distinct curve profiles that the software can easily differentiate (Brunstein, 2014). SNP may be studied to determine their impact on a particular gene or may be utilized to provide associations with genes that play a role in diseases or chronic medical conditions. One

such example of this is a common polymorphism, rs3816527 that has shown to be linked with an increased susceptibility of migraines in a human study (Zandifar, Iraji, Taherium, Tajaddini, & Javanmard, 2015).

A comparison study was performed by Migheli et al. (2013) using two markers linked with tumorigenesis, APC and CDKN2A. They performed this study to ascertain if Methylation-Sensitive High Resolution Melt (MS-HRM) would yield estimates corresponding with results obtained by pyrosequencing, the current gold standard for DNA methylation recognition. Following the completion of the experiments, the methylation results obtained by MS-HRM were mathematically converted to obtain an estimated methylation percentage, and statistical analysis was run using MedCalc software with statistical significance accepted at $p < 0.05$. The resulting methylation values were compared between the two methods, and the outcomes indicated that both MS-HRM and pyrosequencing distinguished similar patterns of methylation. The correlation coefficient, $r = 0.98, p < 0.0001$ shows that the two methods have a strong similarity in ability to identify methylation percentages (Migheli et al., 2013).

Recently, a study was performed that examined the possibility of identifying a VNTR locus D1S80 in humans through High-resolution melt analysis. Samples of three different common allele types were amplified via PCR and then ran through a Rotor-Gene 6000 for the HRM. The results showed that the three allele types: 18/18 homozygous, 18/24 heterozygous, and 24/24 homozygous were all differentiated from one another. These differences were possible because of the sensitivity of HRM analysis to discriminate slight variants in temperature that are associated with large amplicons that are similar. In addition, the software was also able to classify unknown alleles correctly

as part of one of the three previously established allele groups. This illustrated that HRM analysis can be used to examine a large microsatellite, an application that was not previously explored. The authors also concluded that the instrumentation for HRM analysis is "portable, less expensive, a single-tube assay, and easy to use" and could be used "to study their potential use in human identification" (Pomeroy, Balamurugan, Wong, & Duncan, 2014, p. 3026).

Another study, performed by Hanson and Ballantyne (2014) aimed to identify body fluid by RNA profiling-based multiplex run with HRM Analysis. The aim of this study was to minimize the cost and time involved compared to mRNA profiling. Six different tissue types were studied and the results showed that the three different assays showed sufficient differences in the two tissue types for the markers tested. The blood/menstrual blood assay had the closest overlap but was still deemed sufficient for comparison. Overall, this study confirmed HRM is a more cost-effective testing method than others such as capillary electrophoresis or quantitative real-time PCR (qRT-PCR) that use expensive primers or probes. In addition, HRM requires less time for analysis because the PCR and HRM processes are combined. Lastly, the study reviewed that more markers can be tested for in a single reaction whereas qRT-PCR only can test 2 or 3 (Hanson & Ballantyne, 2014).

CHAPTER III

MATERIALS AND METHODS

Sample Collection

DNA from blood, sperm, vaginal epithelial cells, epithelial skin cells, and buccal samples, collected from volunteers for an earlier project, were used. Each of the 14 samples were tested: blood, sperm, vaginal epithelial cells, epithelial skin cells, and buccal samples. All samples were provided with identification numbers, for confidentiality reasons.

Sample Extraction and Modification

The DNA samples available in the lab were extracted using standard organic extraction methods. After each sample was separated from the substrate it was collected on, it was placed in individual 1.5mL tubes and incubated overnight at 56℃ with 400μL of stain extraction buffer and 10μL of proteinase K. The same protocol was used for all sample types except semen samples. 25μL of semen sample is mixed with 150μL of TNE (a mixture of Tris-HCL, NaCl, and EDTA), 50μL of 20% Sarkosyl, 40μL of 0.39M dithiothreitol (DTT), 150μL water, and 10μL of proteinase K and incubated at 56℃ overnight. The samples then went through the same remaining steps to complete the DNA extraction. 500 μL of phenol/chloroform/isopropanol alcohol was added, mixed and centrifuged at 13,000 rpm for 10 minutes. Next, the aqueous layer was pipetted into an Amicon Ultra centrifugal filter devices (Millipore Corporation, Bedford, MA) and centrifuged at 5,000 rpm for 10 minutes. The samples were then washed with Tris-EDTA (TE) buffer at least four times to remove any impurities. After this step, the DNA samples were transferred to new 1.5 mL tubes and stored frozen until use.

Following the extraction procedure, the samples were quantified through gel electrophoresis using 1% agarose with ethidium bromide and visualized with UV light. $1\mu L$ of the sample was mixed with $2\mu L$ of loading dye and pipetted into the wells of the 1% agarose gel. The gel was covered with 1x TAE (Tris/Acetic acid/EDTA) buffer and the gel was electrophoresed for 20 minutes at 120V. Following the gel electrophoresis the gel was visualized in an UV transilluminator and photographed. This allowed the quantitation of the sample and also to check the quality.

All extracted DNA underwent bisulfite conversion using the EpiTect® Bisulfite Kit according to manufacturer's recommendations (Qiagen Inc. Valencia, CA). First, the samples were brought to room temperature for the bisulfite reaction set up. DNA protect buffer, Bisulfite solution, and RNase-free water were added to the samples in a $200\mu L$ tube for a total reaction volume of $140\mu L$. The samples were vortexed and placed in the thermal cycler. The temperature cycling began with the denaturation step at 95℃ for 5 minutes followed by an incubation step at 60℃ for 20 minutes, and these two steps of denaturation followed by incubation were repeated once more.

Following the conversion of the samples, a clean-up of the converted DNA was performed. This process removed impurities from the bisulfite conversion. To each sample, $310\mu L$ of loading buffer (Buffer BL) was added and the tube was vortexed and centrifuged briefly at 13,000 rpm. $250\mu L$ of 96% ethanol was added, with 15 seconds of pulse vortexing. Next, the samples were transferred to $MinElute^{®}$ DNA spin columns and centrifuged for 1 minute at 13,000 rpm, the flow-through discarded. $500\mu L$ of wash buffer (Buffer BW) was added, centrifuged at maximum speed, and flow-through discarded. $500\mu L$ desulfonation buffer (Buffer BD) was added and the tubes were

incubated for 15 minutes at room temperature. After the incubation period, the spin columns were centrifuged at 13,000 rpm for 1 minute and the flow-through was discarded. Next, 500μL of Buffer BW was added, the test tube was centrifuged at maximum speed, and the flow-through discarded. This step was repeated once more. Then, 250μL of 96% ethanol was added to the spin columns and centrifuged for one minute at 13,000 rpm. The spin columns were then placed into new 2mL test tubes and centrifuged at 13,000 rpm for one minute, and then placed with their lids open, in a heating block at 60℃ for 5 minutes. The spin columns were then transferred to new 1.5mL tubes, and 20μL of Eluting buffer was placed directly on the center of the spin column. The spin columns were incubated at room temperature for one minute and then centrifuged at maximum speed for one minute to elute the DNA. Again, 20μL of the eluting buffer was added to the column, incubated at room temperature for one minute, and centrifuged at maximum speed. The samples were stored in a -20℃ freezer until they were ready for the next step. The converted human DNA samples were amplified using locus specific primers, and quantitated with 2% agarose gel before they underwent HRM using a Rotor-Gene 6000 (Qiagen Inc., Valencia, CA) to determine melting temperature.

Loci Selection and Assay Design

In prior studies DDX4 and B_SPTB_03 markers were shown to have unique methylation patterns for sperm samples as compared to vaginal epithelial cells, skin epithelial cells, buccal cells, and blood. Pyrosequencing using a Pyromark® Q24 Pyrosequencer showed that the sperm tissue had hypermethylation in in DDX4 while the other tissues had hypermethylation and vice versa in B_SPTB-03 marker.

The two markers used for blood identification, cg-06379435 and cg-08792630 were “strongly hypermethylated in the target body fluid compared with the other body fluid types” (Park et al., 2014, p. 149). This methylation information was used to analyze the samples using HRM study. The primer sequences used for each marker are listed in Table 1.

Table 1

Primer Sequences and Target Sequence for 2 Sperm and 2 Blood Markers

Marker	Primer Sequences	Target Sequence
B_SPTB_03	FP: 5'GTTGTTTTTGGTTTTTAGGAGAGT 3' RP: 5'AATCCCAACCAACTACTTCCTCCA 3'	5' CGGGGTGTTTTTCGCGGCGGGGCG 3'
DDX4	FP: 5' GGTTTAATAGGTTATTTGGTTATGAGG 3' RP: 5' TCCCCCCTCCACTTCTAACC 3'	5' CGTCGTTATAGGGGTTCGAACGTTAG CGTTTAGGGAATTCG 3'
cg-06379435	FP: 5' AAGAAAAATGTAATTTATTTTTGGGTAT 3' RP: 5' AACAACTATCTCTAATTAAACCCTAC 3'	5' CGGGATAATCGGTGGAATTTTAGGCG TGGGACGGTTGTCG 3'
cg-08792630	FP: 5' GGGTAGTAATGGT TTTTATTTGTAAATGAA 3' RP: 5' CACACCTCTCTCCCTACC 3'	5' CGAAGAGAGATTTTTTATCGTTTTTTAT TATTTTTTTGGGGTACGGGATACG 3'

Note: FP: Forward Primer, RP: Reverse Primer

Custom designed primers were obtained from Integrated DNA Technologies (IDT), Inc. (Coralville, IA). The DDX4 marker contains 6 CpG sites, whereas B_SPTB_03 contains 5 CpG sites. The cg-06379435 marker contains 5 CpG sites, and the cg-08792630 marker contains 4 CpG sites. The expected amplicon sizes and the number of CpG sites to be analyzed for each marker are given in Table 2.

Table 2

Amplicon Size and Number of CpG Sites in Each Marker

Marker	Number of CpG Sites	Amplicon Size
B_SPTB_03	5	90 bp
DDX4	6	96 bp
cg-06379435	5	98 bp
cg-08792630	4	103 bp

Note: bp: base pairs

Template Preparation for HRM

All samples were amplified at the 4 markers: DDX4, B_SPTB_03, cg-06379435, and cg-08792630. Bisulfite converted DNA was added to the primer set consisting of a forward and reverse primer for each marker, and 2X master mix for a total volume of $20\mu L$ as shown in Table 3.

Table 3

Contents of Template Preparation for PCR

Content	Volume
Master mix (2X)	10 μL

Table 3 (continued).

Content	Volume
Forward Primer (25µM)	0.5 μL
Reverse Primer (25µM)	0.5 μL
Bisulfite Converted DNA/water	9 μL
Total Reaction	20 μL

Once the samples were prepared for PCR, the samples were briefly centrifuged, and then were subjected to PCR, consisting of an initial incubation step at 95℃ for 15 minutes, a denaturation step at 94℃ for 30 seconds, annealing for 30 seconds with different temperatures depending on the assay from 50℃ to 53℃, and primer extension at 72℃ for 30 seconds ending with a final extension step of 72℃ for 10 minutes. All the samples underwent 35 cycles of PCR.

Next, the samples were quantitated using 2% agarose gel with ethidium bromide and visualized with UV light. $1\mu L$ of the sample was mixed with $2\mu L$ of loading dye and pipetted into the wells of the 2% agarose gel. The gels were electrophoresed for 15 minutes at 120V. Following electrophoresis, the gels were photographed under UV light.

High Resolution Melt

The Rotor-Gene 6000 (Qiagen Inc., Valencia, CA).was used for the HRM experiment. Immediately following PCR amplification, 1µl of EvaGreen® dye was added to the samples and were placed in the Rotor-Gene and HRM was initiated. The samples were heated at 65℃ and raised to 90℃ at 0.5℃ increments that held for 4 seconds at each temperature. As the temperature rose, the double stranded DNA was

denatured. The rate at which the samples denatured was dependent upon several factors including GC content of the amplified region and temperature. As the DNA began to denature, the intercalating dye that was bound to the DNA disengaged and cease to fluoresce. The instrument measured the fluorescence and Rotor-Gene software plotted the fluorescence versus temperature in a graph. Reagent blanks were used to ensure there was no contamination in the reagents.

CHAPTER IV

RESULTS

For each marker, 14 samples were amplified for every tissue type. However, not all samples amplified to create the target product. This was determined by the presence of the right size band in an agarose gel (Figure 1).

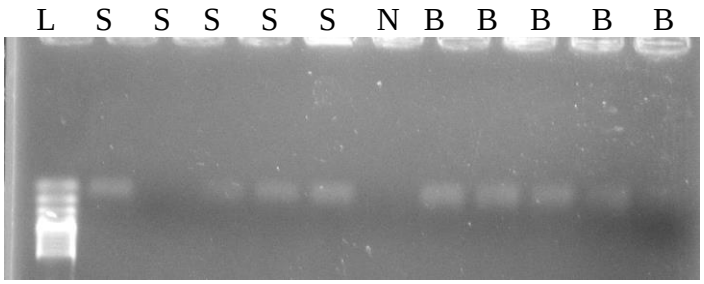

Figure 1. Image of a 2% Agarose gel showing PCR products of sperm and blood samples amplified for the B_SPTB_03 marker. L= 100bp ladder, S=sperm sample, B=blood sample, N=negative control

Sperm Markers

B_SPTB_03 marker

A total of 65 samples were amplified for the marker B_SPTB_03. Normalized curves were created for each run in this experiment. The normalization curve for B_SPTB_03 marker shows distinct pattern for sperm samples compared to the other tissues (Figure 2). For ease in comparing results, the colors green, red, blue, yellow, and orange were consistently used in this experiment to represent the normalized and melt curves of sperm, blood, buccal, skin, and vaginal epithelial (v. epi.) tissues, respectively.

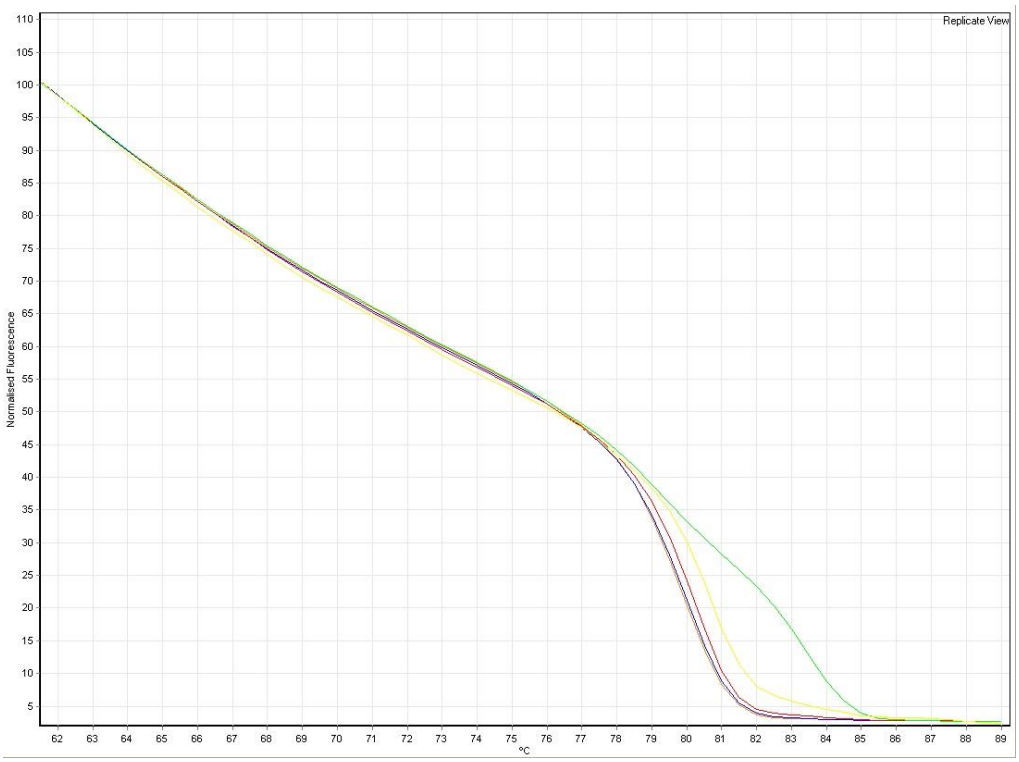

Figure 2. Image of a normalized curve for B_SPTB_03 marker showing the average of the individual samples for each tissue type.

The normalized curve was converted to a melt curve by computer software that takes the derivative slope of the fluorescence over temperature. The average melt curve showing the Tm for each tissue type for the SPTB_03 marker if given in Figure 3.

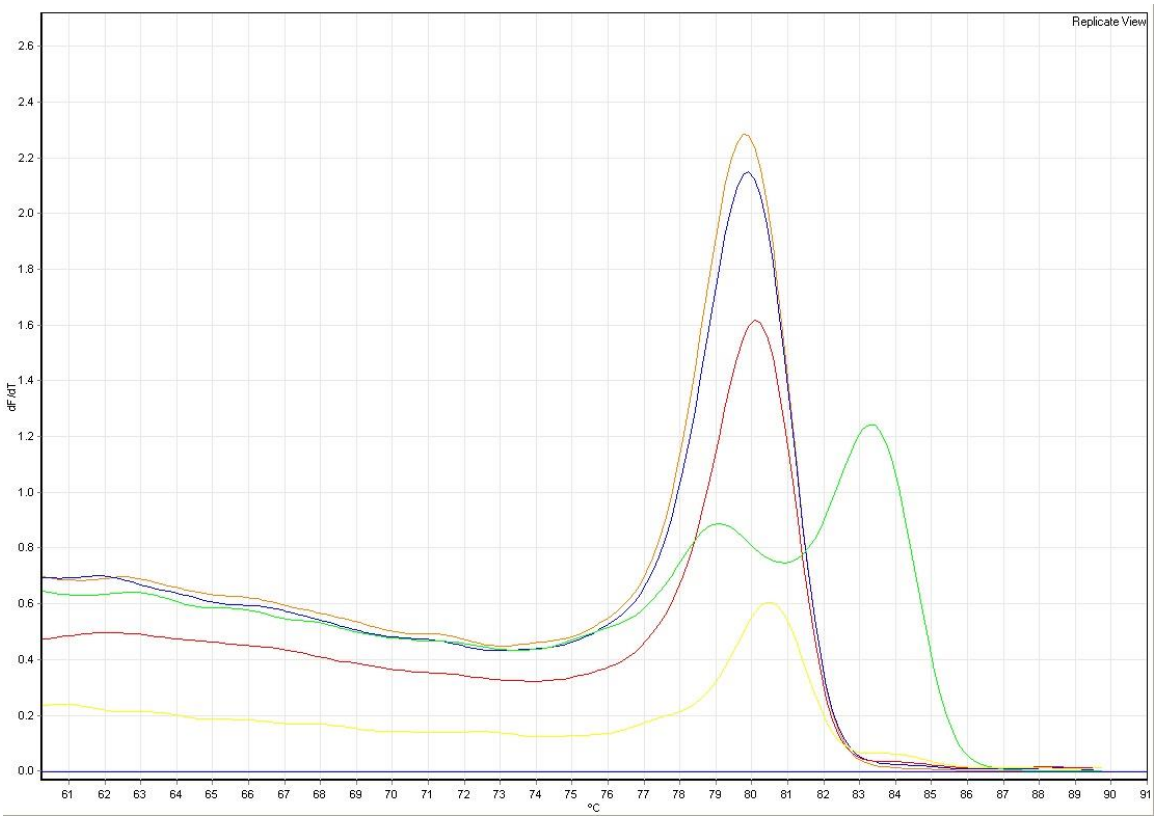

Figure 3. Average melt curves from samples amplified for the B_SPTB_03 marker showing the melting temperature average for the sperm samples (in green) to be approximately three degrees higher than the melting temperature averages of the other tissue types.

For the B_SPTB_03 marker, the sperm samples (n=13) averaged 83.60+/- 0.61℃, whereas the four other tissue types (n=13 buccal; n=12 blood, skin, and v. epi) averaged a temperature of 79.87+/-0.09℃ (Figure 4).

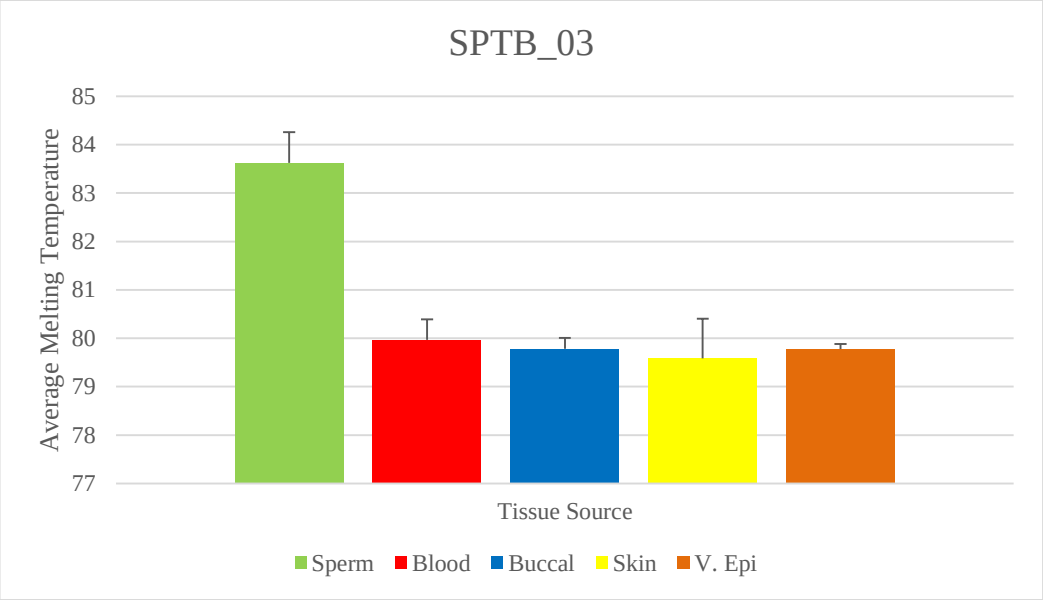

Figure 4. Average melt curve temperatures (°C) with standard deviation for the B_SPTB_03 marker. Individual melting temperatures for the different samples is given in Table 4 (Appendix).

DDX4 marker

In all, 65 samples were amplified with the DDX4 marker. The average melt curve shows distinct difference in the sperm samples compared to the four other tissue types (Figure 5). The melting temperature of sperm samples is lower than the melting temperatures of the four other tissue types tested for the DDX4 marker (Figure 6).

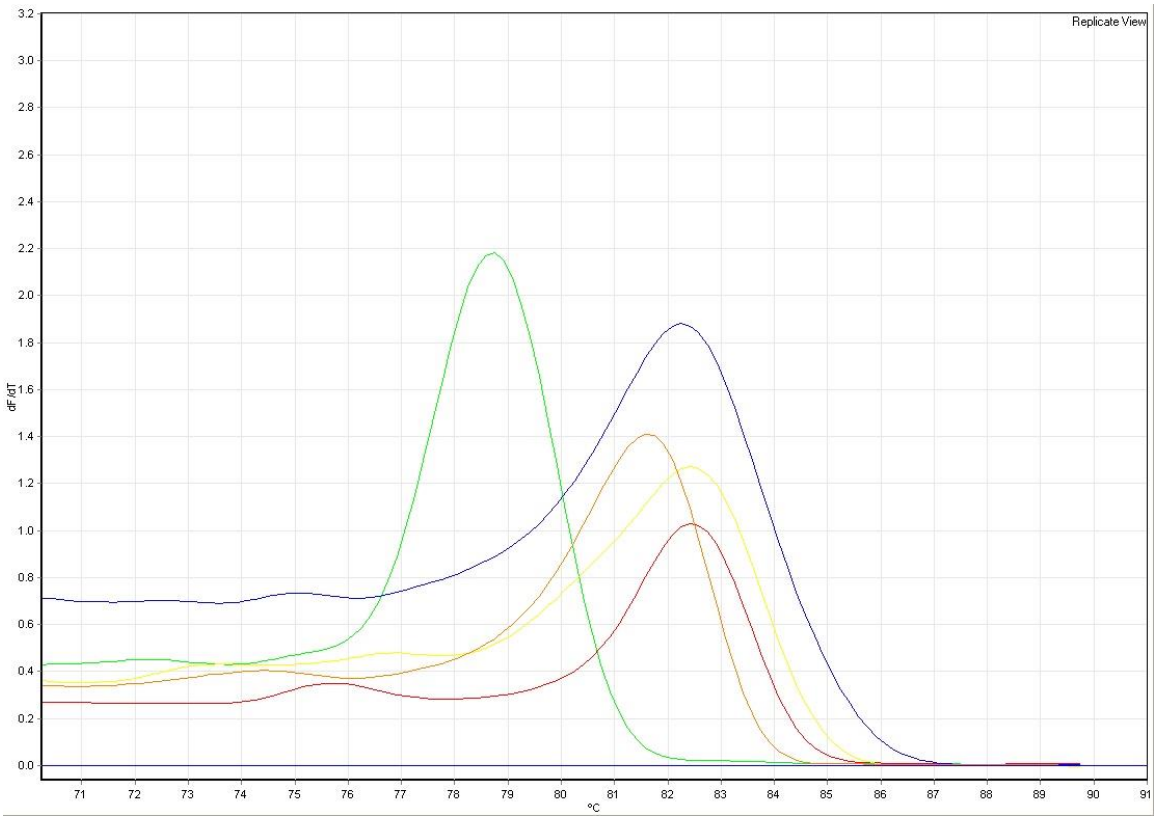

Figure 5. Melt curves from samples amplified for the DDX4 marker showing the melting temperature average for the sperm samples (in green) to be approximately three degrees lower than the melting temperature averages of the other tissue sources.

The sperm samples (n=13) averaged 78.80+/- 0.30℃, whereas the four other tissue types

(n=13 buccal, blood, skin and v. epi) had an average temperature of 82.08+/-0.44℃

(Figure 6).

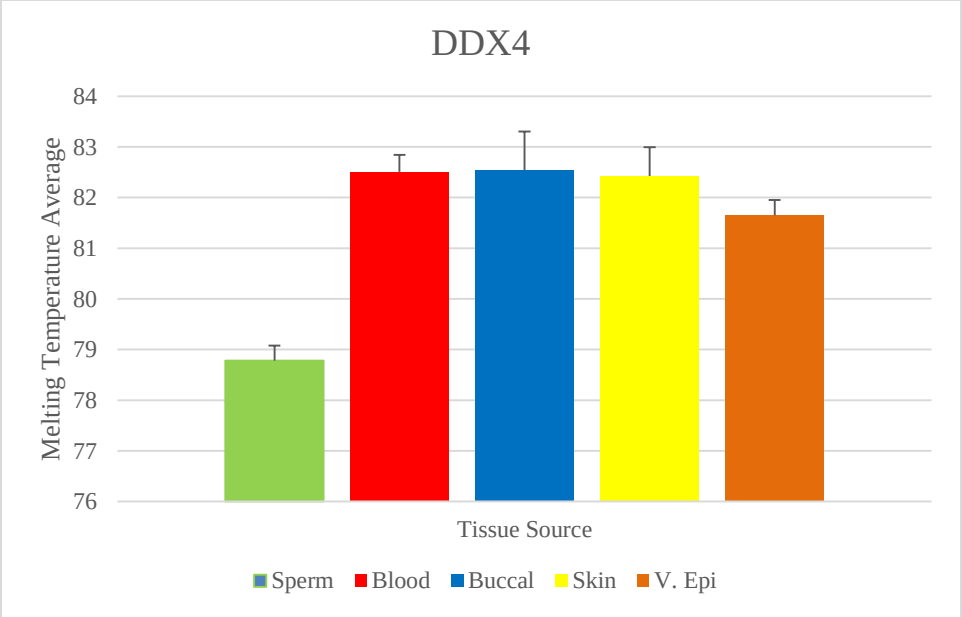

Figure 6. Average melt curve temperatures (°C) with standard deviation of each tissue type for the DDX4 marker. Individual melting temperatures for the different samples is given in Table 5 (Appendix).

Blood Markers

Cg-08792630 marker

The cg-08792630 marker was amplified in 64 samples. The average melt curve shows that the blood samples have a higher melting temperature than the other tissue types studied. (Figure 7).

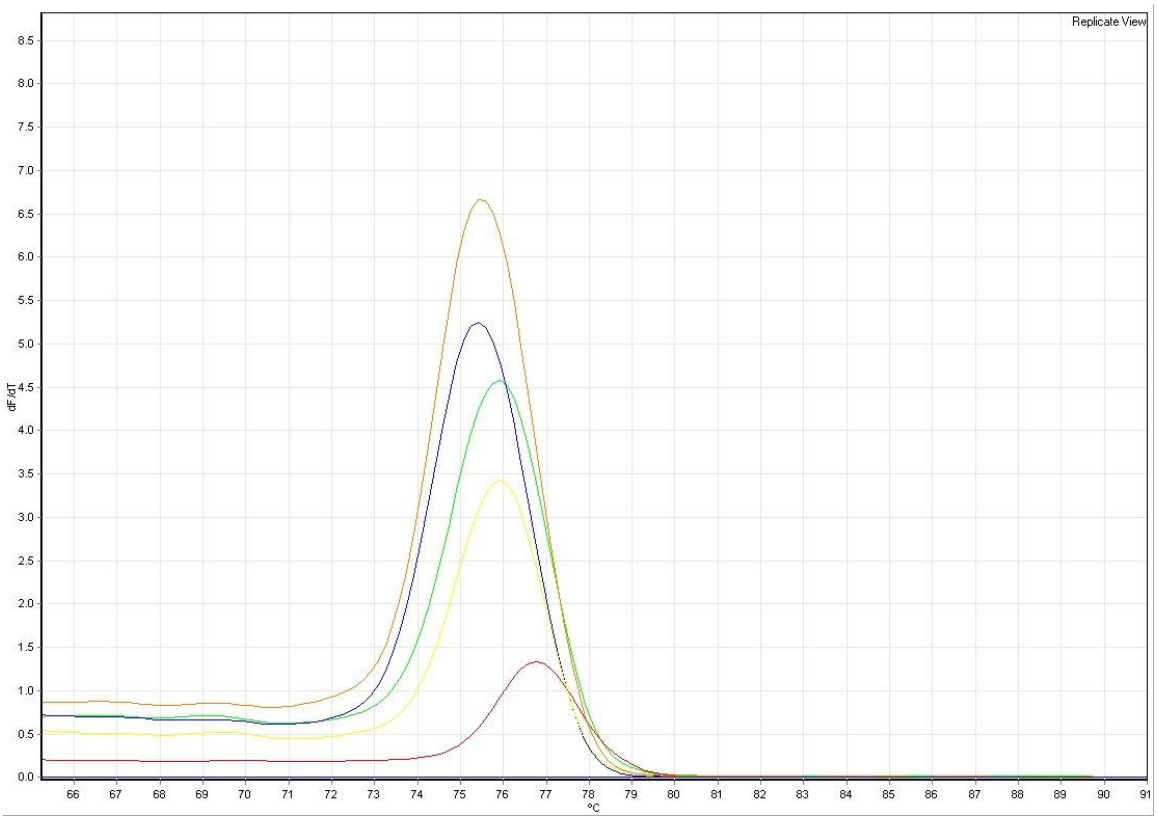

Figure 7. Average melt curves from samples amplified for the cg-08792630 marker showing the melting temperatures for the blood samples (in red) to be approximately one degree higher than the melting temperatures of the other tissue types.

For the cg-08792630 marker, the blood samples (n=13) averaged 76.78+/- 0.28℃,

whereas the four other tissue types (n=13 sperm, skin, and v. epi; n=12 buccal) averaged

75.62+/-0.25℃ (Figure 8).

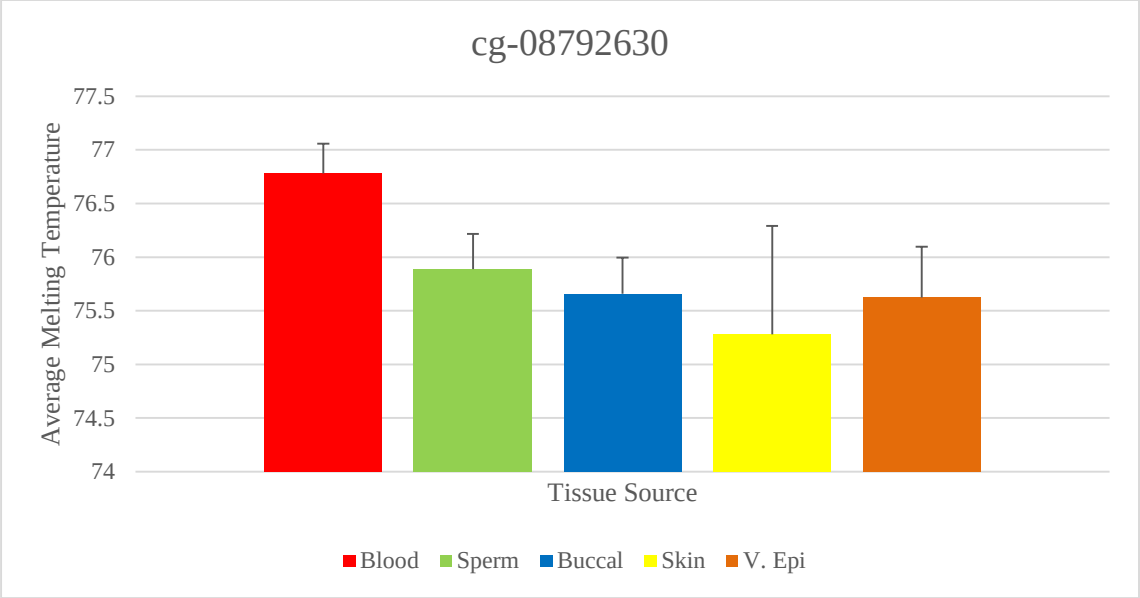

Figure 8. Average melt curve temperatures (°C) with standard deviation for each tissue type for the cg-08792630 marker. Individual melting temperatures for all the samples used is given in Table 6. (Appendix).

Cg-06379435 marker

A total of 63 samples were amplified for the cg-06379435 marker. The average melt curve shows that the blood samples have a higher melting temperature than the other tissue types (Figure 9).

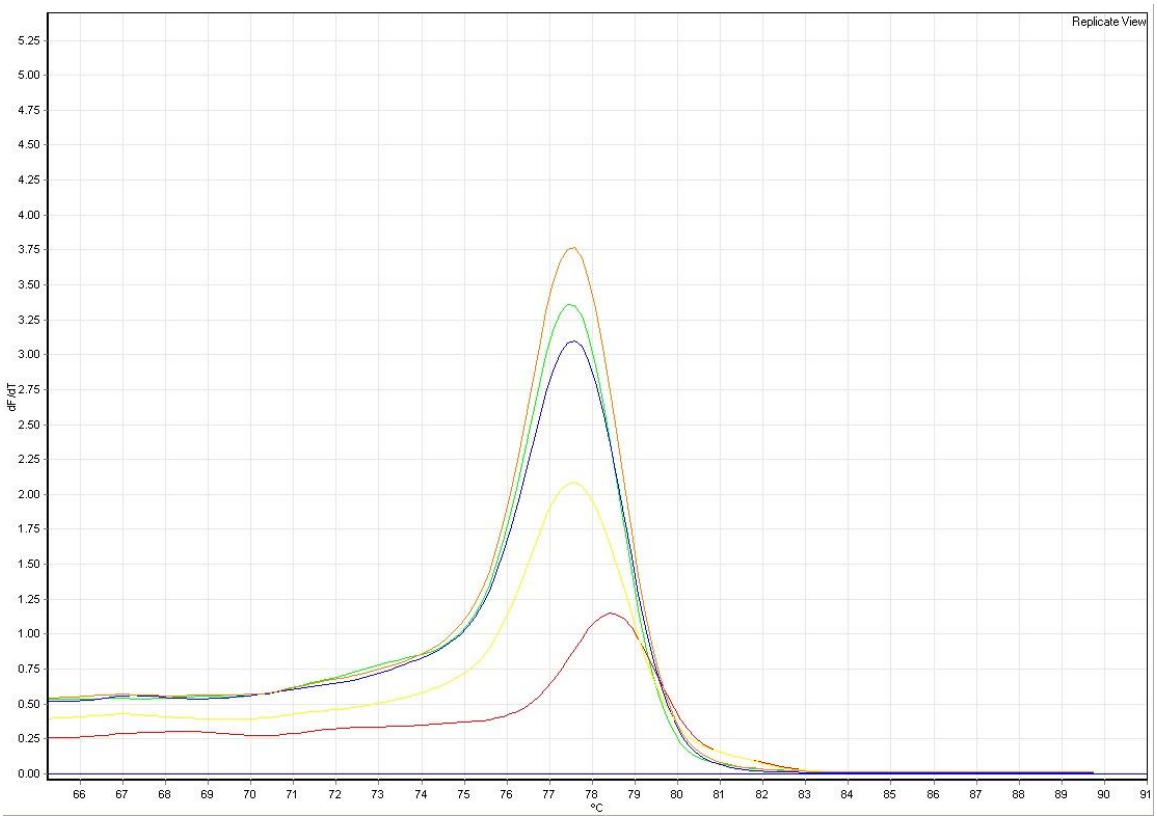

Figure 9. Average melt curves from samples amplified for the cg-06379435 marker showing the melting temperature average for the blood samples (in red) to be approximately one degree higher than the melting temperature averages of the other tissue types.

For the cg-0679435 marker, the blood samples (n=12) averaged 78.48+/- 0.46℃ whereas, the four other tissue types (n=13 sperm, skin, and v. epi; n=12 buccal) averaged a temperature of 77.56+/-0.08℃ (Figure 10).

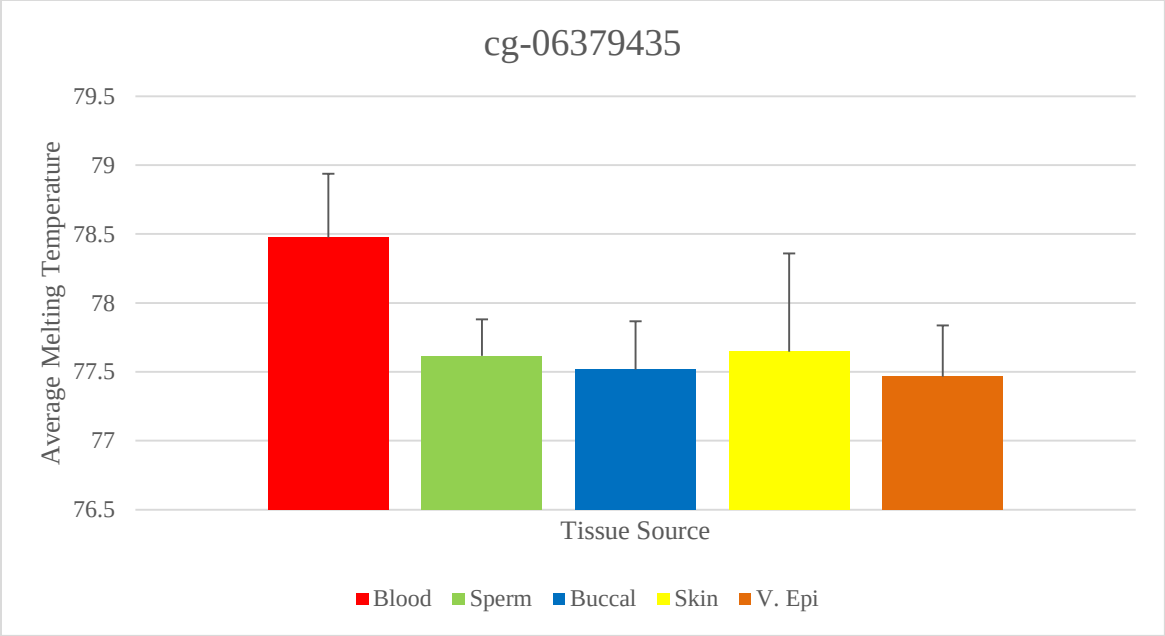

Figure 10. Average melt curve temperatures (°C) with standard deviation for each tissue type for the cg-06379435 marker. Individual melting temperatures of the different samples is given in Table 7. (Appendix).

Amplicon Size and Melt Curve Difference

The amplicon sizes of the first set of experiments ranged from 133 – 262bp in length. When these amplicons were subjected to HRM study, the results were inconsistent; in one set of samples, the sperm DNA was distinguished from the rest of the tissues tested while in the next set of samples there was no clear separation of melt curves between the tissues. Subsequently, when the primers were reengineered to produce an amplicon size of 103 bp or smaller, the sperm and blood DNA were separated from other tissues with distinct melt curves.

For the four markers tested, there was a small portion of samples that failed to amplify, which resulted in a variation in the number of samples analyzed per tissue. For all the four markers, the number of samples that produced readable results ranged from 12-13 for each tissue. The failure of samples in a PCR was not specific to one tissue

types, since it was observed that one or two samples failed to amplify from blood, sperm and skin samples.

CHAPTER V

DISCUSSION

Crime scene reconstruction is a vital aspect of forensic science casework, where one can prove or disprove the statement of someone involved in a crime. The same principle can be applied to other areas of forensic casework such as determination of the tissue source of a DNA sample. In forensic biology, the determination of the tissue source of a DNA is as important as the determination of the DNA profile of a crime scene sample. For example, DNA transfer from a skin sample may have been caused by a simple handshake, but the transfer of DNA derived from blood may have a different story. That is why it is important to determine the tissue source of a DNA sample in a forensic casework. Older technologies such as the Kastle-Meyer test are still used as a presumptive test for blood and the AP spot test or the Acid Phosphatase enzyme test is used as a presumptive test for seminal fluid. A more demanding answer may be required in some instances and the analysis of DNA can provide the answer. Recently several studies have been reported on the use of tissue specific differential methylation levels in certain markers in the DNA (Lee et al., 2012; Park et al., 2014; Balamurugan et al., 2014). Methylation is an epigenetic change that is associated with gene control and expression. In most instances, high levels of methylation in the control region of a gene are linked with lack of gene expression, and low levels of methylation are linked with gene expression, and these expressions are found to be tissue specific. Certain markers are hypermethylated or hypomethylated due to the gene expression they are controlling. These have been proved to be very valuable in the determination of a tissue source of a DNA sample. Even though the current technologies for the serological identification of a

tissue are used in the crime labs, newer technologies are on the rise. A new technology named High Resolution Melt (HRM) analysis is gaining popularity, but not very many reports have been published (Nguyen, McKinney, Johnson, Roberts, & Hardy, 2012; Pomeroy et al., 2014).

The epigenetic markers B-SPTB-03 and DDX4 were found to differentiate sperm from other body fluids (blood, buccal, skin and v. epi.) while markers cg-06379435 and cg-08792630 were found to differentiate blood from other four tissues based on the methylation levels of each tissue. In this study four epigenetic markers (B_SPTB_03, DDX4, cg-06379435, and cg-08792630) were chosen to study if the HRM technology can be used to distinguish the different tissue types based on the level of methylation detected in each tissue.

Differences in Melt Curves Between Tissue Types

Sperm markers: B_SPTB_03 and DDX4

In this study, the DNA melt curve of two markers, B_SPTB_03 and DDX4 have shown a three degree difference in melting temperature (Tm) in sperm samples when compared to samples originating from the other four tissues tested. The B_SPTB_03 marker in the sperm DNA is found to be hypermethylated compared to other tissues (Balamurugan et al., 2014), and the CpG sites in the sperm samples retain the 'C' in the CpG site, while the 'C' in the CpG site of other tissues were converted to 'U' during the bisulfite conversion, and subsequently the 'U' is converted to a 'T' during the PCR process. The presence of a 'C' increases the melting temperature of the DNA while the presence of a 'T' lowers the temperature (Figures 3, 4). For the DDX4 markers this is vice versa. The sperm DNA samples are hypomethylated for this marker while the other

tissues are hypermethylated. This leads to the fact that DNA that is hypermethylated has a higher Tm compared to DNA that is hypomethylated. By looking at the melt curve and the melt temperatures of the B_SPTB_03 and the DDX4 marker, one can easily note that there is 3°C difference between the sperm and other tissues (Figures 4, 6). This melting temperature characteristic can be successfully used as a diagnostic tool to differentiate DNA originating from a sperm sample versus a non-sperm sample. The melt curve is very characteristic to a DNA sequence and even a single base change between two alleles or samples can be differentiated (Bates, 2014). Pomeroy et al. (2014) has studied several repeat sequences of the D1S80 marker and have proved that DNA samples with identical sequence have identical melting temperatures (with a very small standard deviation) even if those sequences are derived from different samples.

Blood markers: cg-06379435 and cg-08792630

Park et al. (2014) have reported that the two markers cg-06379435 and cg-08792630 have higher level of methylation on a specific CpG site in blood compared to other tissues. Additional studies by Balamurugan et al. (unpublished data) confirm this report not only to the specific site studied by Park et al. but several other adjacent CpG sites as well. Park et al reported that blood and other tissues had a difference of about 30-40% in their methylation levels.

The above two markers were chosen to study the melt curve difference and for the marker's usefulness in differentiating blood from other tissues based on the methylation pattern. The melt curve difference of blood samples compared to other tissues was observable but not as pronounced as in the other two sperm markers. Both the cg-6379435 and cg-8792630 markers were able to differentiate or separate blood from other

tissues based on the normalized curve, melt curve, and Tm differences; however, the Tm of blood samples differs only by 1°C when compared to other tissues (Figures 8, 10). This small difference is due to the fact that the methylation level of blood samples are about 40% and other tissues showed a methylation level of 10% or below. This leads to a methylation difference of only 30% between blood and other tissues. Such a difference can be used to differentiate blood form other tissues if one is using the pyrosequencing technology for quantitative methylation levels, but not with the HRM technology.

Melt Temperature Peaks

Some melting temperature peaks present in the markers tested appear to be broader than other melting temperature peaks. This observation is more noticeable in the blood markers that were tested. This phenomenon can be explained by the instrument, the settings, and the markers tested. The settings that were chosen for this experiment were such that a reading was taken with every 0.5 degree raise in temperature. Some tissue samples contained one species of DNA that began to denature at one temperature and another species of DNA continued to denature at the next raised temperature. Therefore, the DNA in the tissue samples were denatured in a close proximity of time or in an extended temperature interval. The instrument reads levels of fluorescence, and creates a curve that corresponds to this extended temperature interval, and this caused some peaks to appear broader than other peaks.

Some of the melt curves have a higher fluorescence such as buccal, sperm, and v. epi. samples. This is due to the higher concentration of DNA in these samples when compared to blood and skin samples. The higher fluorescence does not alter the melting temperature, and therefore does not alter the results.

Effect of Amplicon Size on Melt Curve

Amplification of the right target segment is vital in utilizing the HRM technology. In the initial experiments, the primers that were used for the pyrosequencing were used for amplifying the target segments. The amplicons that were produced for all the markers ranged from 133 – 262bp in length. When these amplicons were subjected to HRM study, in very few experiments there was differentiation of one tissue from another while there was no clear separation between the tissues majority of the times. Determination of differentiation between the tissues was based off the software image and statistical analysis of the individual melt temperatures. When these samples and markers were tested elsewhere in another laboratory with another Rotorgene instrument, the same problem was encountered. This raised the suspicion that the amplicon length may be too large. Subsequently, the primers were reengineered to produce an amplicon size of 103 bp or smaller. When those reengineered primers were used for the amplification, followed by HRM, the sperm and blood DNA can be separated from other tissues with distinct melt curves.

The percent difference in sequence between the two samples is critical in HRM analysis. As mentioned above, the length of the amplicon proved to be an issue in getting distinct melt curves for different tissues. For example, in the SPTB_03 marker, the original amplicon size was 260 bp with seven CpG sites. As was proven in previous study by Balamurugan et al. (2014), a sperm sample with 100% methylation at all seven sites would have a 2.69% (7/260 x 100) difference compared with other tissues with 0% methylation in the seven sites. When the amplicon size is reduced to 90bp, the number of CpG sites decreased to five due to the reengineered primers. Even with that decrease, the

percent difference between the sperm and the other tissue types would be 5.5% (5/90x100). Although only five CpG sites were present in the smaller amplicon, the melting temperatures for the sperm samples were distinct compared to other tissues. This leads to the fact that the percentage difference in sequence (not the number of differences) between two tissues makes a big difference in discriminating one tissue from another; the higher the percentage difference, the higher the difference in melting temperature.

Future Research

HRM is a biological application that has not been utilized for its full potential, especially in the area of forensic science. Future research could experiment with more markers for differentiating individual tissue types. In addition, studies that examine the efficiency of different dye/master mix combinations could be useful as well as determining a limit on PCR product size for adequate amplification in methylation based studies.

Furthermore, future research may provide more information if HRM is used to determine and confirm methylation differences for other applications such as differentiation between identical twins, age groups, and phenotypic variance. Other potential areas could be exploring genetic and epigenetic differences in athletes and non-athletes (Saunders, Posthumus, O'Connell, September, & Collins, 2015).

Conclusion

It has been observed that HRM can differentiate DNA originated from different tissue types. The differentiation primarily depends on three factors: (1) the number of CpG sites available for study; (2) the amount of methylation difference between the two

tissues, and (3) the percentage difference in the DNA sequence between two tissues. If there are multiple CpG sites available for study, it leads to an increased difference between the two tissue types. On the other hand, the higher the % methylation difference between the two tissues, the higher the melting temperature difference and better separation of the melt curves. The sperm samples are clearly differentiated from the other tissues for the markers B_SPTB_03 and DDX4 based on the melt curve whereas the blood was not as clearly differentiated from other tissues for the markers cg-6379435 and cg-8792630.

APPENDIX A

Table 4

Individual Melt Curve Temperatures (°C) *for B_SPTB_03*

	Sperm	Blood	Buccal	Skin	V. Epi
	83.15	79.75	80.00	80.40	79.90
	83.50	80.25	80.00	80.90	79.75
	83.75	80.35	79.75	79.25	79.85
	83.50	79.90	80.00	78.90	79.75
	83.60	79.90	79.75	79.25	79.75
	85.50	80.10	79.85	80.65	79.60
	83.75	80.15	79.25	79.00	79.60
	83.50	80.15	79.90	78.90	79.85
	83.25	80.35	79.65	79.00	79.90
	83.00	78.85	79.85	81.00	79.75
	83.35	79.90	79.65	80.60	79.85
	83.60	80.40	80.10	80.75	80.00
Average	83.60	80.00	79.81	79.88	79.80
Standard Deviation	0.612	0.42	0.22	0.88	0.12

Table 5

Individual Melt Curve Temperatures (°C) *for DDX4*

	Sperm	Blood	Buccal	Skin	V. Epi
	79.00	82.85	82.25	82.25	82.00
	78.65	82.85	82.00	83.75	81.90
	78.35	82.50	83.50	82.75	81.00
	78.75	82.35	81.90	82.15	81.35
	78.40	82.00	83.25	82.00	81.75
	78.50	82.75	82.35	81.90	81.50
	79.10	82.60	81.90	82.90	81.35
	79.25	82.75	82.25	82.10	81.85
	79.00	82.25	83.75	81.50	82.00
	79.15	82.25	82.00	82.25	81.40
	78.75	83.10	83.75	82.65	81.85
	78.75	82.10	82.60	82.60	81.60
	78.50	82.25	81.50	82.75	81.85

Table 5 (continued).

	Sperm	Blood	Buccal	Skin	V. Epi
Average	78.78	82.51	82.63	82.40	81.65
Standard Deviation	0.30	0.34	0.76	0.56	0.31

Table 6

Individual Melt Curve Temperatures (°C) *for the cg-08792630*

	Blood	Sperm	Buccal	Skin	V. Epi
	76.75	76.00	75.35	75.75	75.35
	76.65	75.65	75.50	76.40	75.25
	76.50	75.50	75.25	75.60	75.85
	76.85	76.40	75.50	76.10	75.60
	77.00	76.00	75.15	76.15	75.40
	76.50	75.25	75.50	76.35	76.35
	77.25	75.90	75.75	74.10	76.35
	76.50	75.85	75.85	76.10	76.00
	76.75	75.75	76.00	74.10	75.65
	76.40	76.25	76.15	75.70	75.90
	76.90	76.35	76.15	74.85	74.85
	76.85	75.75	75.75	74.15	75.65
	77.25	75.90		73.50	74.90
Average	76.78	75.89	75.66	75.28	75.62
Standard Deviation	0.28	0.33	0.34	1.01	0.48

Table 7

Individual Melt Curve Temperatures (°C) *for the cg-06379435*

	Blood	Sperm	Buccal	Skin	V. Epi
	78.90	77.60	77.65	77.75	77.35
	78.50	77.35	77.50	77.50	77.90
	78.90	77.40	78.15	77.40	77.35
	78.25	77.40	77.50	78.00	77.40
	78.40	77.90	77.50	78.60	77.75
	78.25	77.75	77.00	77.00	77.25
	78.50	77.50	77.50	78.60	77.35
	77.60	77.60	77.00	77.25	77.75
	79.40	78.15	77.90	77.65	77.40
	78.35	77.85	77.15	77.90	77.90
	78.00	77.75	77.50	78.25	77.40
	78.65	77.15	77.85	77.60	77.75
		77.60		75.90	76.50
Average	78.48	77.62	77.52	77.65	77.47
Standard Deviation	0.46	0.27	0.35	0.71	0.37

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